Regulation of Muscarinic Acetylcholine Receptor Expression and Function[a]

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Muscarinic acetylcholine receptors (mAChR) are members of the superfamily of G-protein-coupled cell surface receptors that are characterized by the presence of seven putative transmembrane domains. Other members of this family include alpha- and beta-adrenergic receptors, the opsins, olfactory receptors, the dopamine receptors, and the receptors for many neuropeptide and glycoprotein hormones (see Dohlman *et al.*[1] for review). The mAChR themselves represent a small gene family: the genes for five subtypes of mAChR have been identified by molecular cloning. Muscarinic receptors are present in both the central and peripheral nervous systems, cardiac and smooth muscle, and various exocrine glands (see Nathanson[2] and Bonner[3] for review). Muscarinic receptors produce functional responses either by regulating the activity of enzymes such as adenylyl cyclase (AC) or phospholipase C (PLC) which produce intracellular second messengers, or by regulation of ion channels such as potassium and calcium channels. The m1, m3, and m5 receptors couple preferentially to stimulation of PLC, and the m2 and m4 receptors couple preferentially to inhibition of AC. However, the specificity of mAChR functional coupling is dependent both on levels of receptor expression and on the cell type in which a given receptor or subtype is expressed (see Tietje & Nathanson[4] and references therein). Muscarinic receptor number can be altered in response to sustained agonist exposure. Short-term agonist exposure (s to min) causes a rapid removal of mAChR from the cell surface (sequestration) whereas agonist exposure for longer periods of time (h) causes a decrease in total receptor number (down-regulation). In this report we describe studies on the functional analysis of mAChR coupling mechanisms using a reporter gene system for the detection of mAChR-mediated changes in intracellular cAMP levels. We also demonstrate that a tyrosine residue in the intracellular carboxyl tail of the m2 receptor is important in agonist-mediated down-regulation but is not required for agonist-mediated functional responsiveness or receptor sequestration. Finally, we also demonstrate that long-term agonist activation of mAChR in chick heart cells leads to decreases in the level of mAChR mRNA levels.

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REGULATION OF cAMP-MEDIATED GENE EXPRESSION BY MUSCARINIC RECEPTORS AND G-PROTEINS

We used a luciferase reporter gene under the transcriptional control of a cAMP response element (CRE) to measure the regulation of intracellular cAMP levels and cAMP-regulated gene expression in response to mAChR activation.[5] Treatment of transiently transfected JEG-3 human choriocarcinoma cells with forskolin, which increases intracellular cAMP levels due to the activation of adenylyl cyclase, results in a greater than 10-fold increase in luciferase activity. Expression of the CRE-luciferase reporter gene in JEG-3 cells is not significantly regulated by changes in intracellular calcium levels. Treatment of untransfected cells with the muscarinic agonist carbachol does not result in an increase in luciferase expression, even though the cells express a low level of endogenous mAChR which mediate a 3–5-fold carbachol-stimulation of phosphatidylinositol turnover. Furthermore, treatment with the calcium ionophore A23187 or with the phorbol 12-myristate,13-acetate, or both together only results in small increases in luciferase expression. Treatment of JEG-3

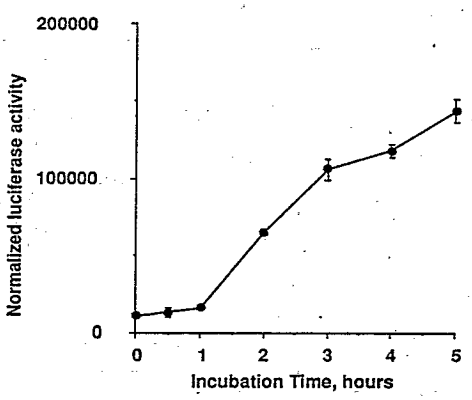

FIGURE 1. Time course of m1-mediated increase in luciferase activity. Two hours are required to detect agonist stimulation of CRE-mediated luciferase expression in m1-transfected JEG-3 cells. Cells transfected with the m1 receptor and the CRE-luciferase reporter gene were treated with carbachol (1 mM) for varying amounts of time prior to harvesting and determination of normalized luciferase activity. Data are the mean of triplicate determinations ± SD. CRE, cAMP response element.

cells transiently transfected with mouse m1 and chick m4 mAChR results in 8–10-fold and 3–5-fold increases, respectively, in luciferase activity. In order to determine the amount of time necessary to detect changes in luciferase expression, m1 transfected cells were incubated with carbachol for varying amounts of time and harvested immediately thereafter (FIG. 1). Increases in luciferase activity are first detectable at the 2-h time point and level off between 3 and 5 h. In order to determine the minimal duration of stimulation required to observe a subsequent increase in luciferase gene activity, JEG-3 cells were treated with either carbachol or forskolin for varying amounts of time, after which the drug was removed and the cells were washed and reincubated with fresh media. All cultures were harvested 5 h from the beginning of the drug treatment period (FIG. 2). Stimulation of luciferase activity mediated by m1 mAChR was easily detectable when the carbachol was removed after only a 5-min incubation and peaked after 3–5 h of incubation. The carbachol-mediated responses are as sensitive to inhibition by cotransfection with a cAMP-dependent protein kinase (PKA) inhibitor peptide or a dominant negative mutant PKA regulatory subunit as is the forskolin-stimulated response. Thus, m1- and

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m4-mediated responses are dependent upon a functional cAMP-dependent protein kinase.

The m1- and m4-mediated increases in luciferase expression are not blocked by pretreatment with pertussis toxin, and the m4 response was potentiated by it. Thus, the G-proteins G_i and G_o are not involved. The most reasonable hypothesis to explain the apparent increase in intracellular cAMP is that the m1 and m4 receptors can activate adenylyl cyclase by interacting with the stimulatory G-protein G_s. Consistent with this hypothesis, both the m1[6] and m4[7] receptors can activate adenylyl cyclase activity in membranes prepared from stably transfected cells.

Atropine treatment of JEG-3 cells transiently transfected with high levels of m4 mAChR, but not m1, causes an elevation in basal levels of CRE-mediated luciferase expression in the absence of agonist.[5] Furthermore, treatment of m4- but not m1-transfected cells with pertussis toxin caused a significant increase in the level of luciferase expression in the absence of agonist. These results suggest that the m4 receptor is spontaneously active and can cause constitutive inhibition of adenylyl

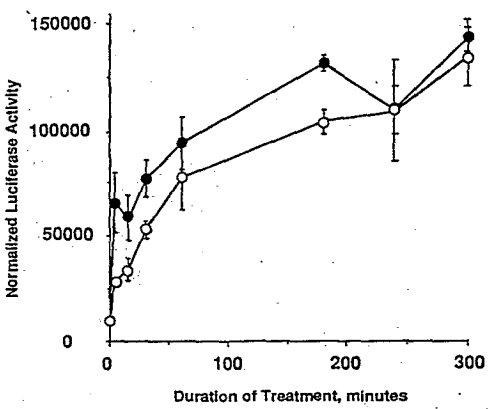

FIGURE 2. Induction of luciferase expression after carbachol or forskolin treatment. Five minutes of carbachol or forskolin treatment are sufficient to detect stimulation of CRE-mediated luciferase expression in m1-transfected JEG-3 cells. Cells transfected with the m1 receptor and the CRE-luciferase reporter gene were treated with carbachol (1 mM; *open circles*) or forskolin (1 μM; *closed circles*) for varying amounts of time (0–5 h), after which the cells were washed and allowed to continue incubation until 5 h after the initial administration of drug. Data are plotted as normalized luciferase activity, and values are the mean of triplicate determinations ± SD. CRE, cAMP response element.

cyclase that is relieved by treatment with atropine. This suggests that the unbound m4 receptor exhibits spontaneous activity and that binding of antagonist induces a change in the conformation of the receptor that prevents it from interacting with G-proteins. This hypothesis is supported by the observation that atropine dissociates mAChR–G-protein complexes in rat heart membranes.[8] Furthermore, electrophysiological evidence indicates that mAChR in the heart may exhibit spontaneous activity that can be blocked by antagonist treatment.[9,10] Our results demonstrate that an antagonist can produce a subtype-specific functional response in the absence of agonist.

Surprisingly, the m4 receptor exhibits little if any agonist-induced inhibition of either basal or forskolin-stimulated luciferase expression at either low or high levels of receptor expression.[5] JEG-3 cells express $G_i\alpha$-1 and $G_i\alpha$-3, but not $G_i\alpha$-2. Cotransfection of $G_i\alpha$-1 or $G_i\alpha$-3 does not alter the m4-mediated response, whereas cotransfection with $G_i\alpha$-2 greatly increases the ability of the m4 receptor to inhibit

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forskolin-stimulated luciferase expression. Thus, the m4 receptor requires $G_i\alpha$-2 for optimal agonist-mediated inhibition of adenylyl cyclase activity.

DIFFERENTIAL ROLE OF CARBOXYL-TERMINAL TYROSINE IN AGONIST-INDUCED DOWN-REGULATION OF THE m2 RECEPTOR

Tyrosine residues located in the cytoplasmic tails of many membrane receptors are necessary for agonist-induced internalization of these receptors via clathrin-coated pits (see Goldman & Nathanson[11] for references). In addition, either of the two tyrosine residues located in the cytoplasmic tail of the β_2-adrenergic (β_2AR) is required for the down-regulation but not the sequestration of receptors in response to agonist.[12] The mammalian m2 receptor has a single tyrosine residue (Tyr-459) in the carboxyl terminal cytoplasmic domain. We have used site-directed mutagenesis to determine the role of this tyrosine residue in the regulation and function of the m2 receptor.[11] We found that substitution of Tyr-459 with Phe, Trp, Ala, or Ile affected neither agonist nor antagonist binding, and did not alter the ability of the m2 receptor to inhibit the accumulation of intracellular cAMP. Although elimination of Tyr-459 did not affect agonist-induced sequestration of the m2 receptor, the sensitivity as well as the rate and extent of agonist-induced down-regulation was attenuated in the Ala, Phe, and Trp mutant m2 receptors. Mutation of the Tyr to Ile resulted in a slight decrease in the sensitivity of the m2 receptor to agonist-induced down-regulation, although the maximal decrease in receptor number was not significantly different from the wild-type receptor.

The attenuation of down-regulation observed with the Phe and Trp mutations indicates that the aromatic side chain of the Tyr residue is not sufficient for down-regulation of the m2 receptor. The similar time courses for agonist-induced down-regulation between the wild-type and Ile mutant suggest that the hydroxyl group of Tyr-459 is not required for agonist-induced down-regulation of the m2 receptor. The results suggest that Tyr-459 is important as part of a structural motif in which subtle changes in amino acid structure or size might perturb the overall structure of this domain. These results are the first to identify a site in the mAChR involved in agonist-induced down-regulation and the first to make mutations that affect the long-term down-regulation, but not the rapid sequestration of the mAChR.

REGULATION OF mAChR mRNA BY mAChR ACTIVATION

Cloning studies have demonstrated that at least two subtypes of mAChR are expressed in the embryonic chick heart, the cm2 and cm4 receptors.[4,13] We found that persistent activation of the mAChR in cultured chick heart cells with the cholinergic agonist carbachol causes significant decreases in the levels of both cm2 and cm4 mRNA. The level of mAChR mRNA was measured by solution hybridization using subtype-specific riboprobes. Treatment with carbachol did not alter the half-lives of either the cm2 or the cm4 mRNAs, indicating that the agonist most likely regulates mRNA levels by regulating the rate of gene transcription.[14] The regulation of mAChR mRNA by the agonist has important functional consequences, as it regulates the rate of reappearance of muscarinic receptor number during the recovery from agonist-induced down-regulation after agonist withdrawal.[15]

The regulation of mAChR mRNA levels most likely results from a change in the level of one or more intracellular second messenger. In chick heart, activation of

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mAChR causes both inhibition of adenylyl cyclase activity and stimulation of PLC activity. Several different approaches were used to test the role of second messenger systems in the regulation of mAChR mRNAs. Embryonic chick heart expresses A1 adenosine receptors, which inhibit AC but do not activate PLC, and angiotensin II receptors, which stimulate PLC but do not inhibit AC. Although activation of each receptor separately had relatively little effect on mAChR mRNA levels, concomitant activation of both of these heterologous receptor pathways resulted in significantly greater decreased mAChR mRNA levels. These results suggest that regulation of both adenylyl cyclase and PLC activities are involved in the regulation of mAChR gene expression by mAChR activation.[14]

We also used pharmacological and biochemical experiments to test if both second messenger pathways were involved in regulation of mAChR mRNA levels following homologous receptor activation.[15] Treatment of cells with pertussis toxin, which blocks coupling of muscarinic receptors to AC but not PLC, blocked the muscarinic receptor-mediated decrease of both cm2 and cm4 mRNA levels. Thus, inhibition of AC activity was required for homologous regulation of mAChR mRNA. Incubation of chick heart cells with the partial agonist pilocarpine, which causes inhibition of AC activity but not stimulation of PLC, resulted in significantly smaller decreases of receptor mRNA levels than agonists such as carbachol that regulate both second messenger systems. Thus, coupling to both the AC and PLC pathways is required for maximal regulation of cm2 and cm4 mRNA levels in response to mAChR activation.

CONCLUSIONS

We showed that the CRE-luciferase assay is a convenient system for the determination of mAChR-mediated changes in intracellular cAMP levels. This system should be useful for the determination of the G-protein coupling specificity of other mAChR subtypes as well as structure-function analyses of G-protein subunits. The identification of a potential down-regulation motif in the m2 mAChR will allow the determination of whether similar sequences have similar roles in other mAChR subtypes. Finally, the identification of regulatory sequences in the mAChR genes will allow determination of the molecular and cellular basis for the regulation of muscarinic receptor gene expression following receptor activation by acetylcholine.

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